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(54) Method for synthesizing procoagulant factor VIII activity.

(57) Disclosed herein is a method for synthesizing procoagulant factor VIII from inactive protein by incubating the protein in the presence of an effective amount for synthesizing procoagulant factor VIII of a manganese salt. The method is useful to synthesize AHF in plasma that has been collected with an anticoagulating agent.

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METHOD FOR SYNTHESIZING  
PROCOAGULANT FACTOR VIII ACTIVITY

1 The present invention pertains to a method of generating factor VIII, or anti-hemophilic factor (AHF) activity from inactive protein.

5 Anti-hemophilic factor (AHF), often referred to as factor VIII, is a protein material present in trace amounts in normal human and animal blood plasma. Factor VIII corrects the coagulation defect of hemophilic plasma and also a functional defect in VonWillebrand's disease  
10 plasma. Factor VIII is defined by its activity which is diminished in patients with hemophilia A. AHF is used for the treatment of hemophilia A (which is defined to be a deficiency of factor VIII procoagulant activity). One unit of factor VIII activity is defined as the amount  
15 in one milliliter of pooled, fresh, normal, citrate-treated plasma.

The basis for therapy of bleeding episodes in patients afflicted with hemophilia involves transfusion of material containing factor VIII procoagulant activity  
20 which temporarily corrects this specific defect. In general, whole blood is not administered to the patient, and its use is limited to restoration of blood volume in instances of severe loss. While plasma with a high factor VIII content may be employed, the preferred  
25 method of treatment involves administration of factor VIII concentrates.

Factor VIII is obtained from whole blood plasma donated by human volunteers. However, because only a small quantity of factor VIII is present in a given volume  
30 of blood, it is important to maximize the recovery of this protein material. The problem is complicated because whole blood is generally collected into anti-coagulant materials such as sodium citrate, sodium oxalate, citrate dextrose, citrate phosphate dextrose, or EDTA (ethylene -  
35 diamine tetraacetic acid). The presence of these anti-coagulants tends to diminish the factor VIII activity present in the collected plasma. These standard anti-

1 coagulant compounds are metal chelating agents and it has  
been postulated that they remove a metal element, respons-  
ible for factor VIII activity, from the plasma. The  
effects of these metal chelating anti-coagulant agents are  
5 cumulative, with the result that factor VIII activity  
constantly diminishes in their presence with the passage  
of time. Thus, if a unit of citrated blood is stored for  
more than a few days, under refrigeration, a substantial  
percentage of the factor VIII activity is lost. Although  
10 the coagulant activity of factor VIII is markedly in-  
creased or activated in the presence of traces of thrombin  
and other proteolytic enzymes such as trypsin, the in-  
creased activity is relatively short-lived. The thrombin  
activation effect cannot be observed in coagulant inactive  
15 material.

In general, whole blood collected from human  
volunteers in the presence of an anti-coagulant. By the  
time the blood is centrifuged, frozen, stored, and used  
for fractionation to manufacture a cryoprecipitate con-  
20 taining factor VIII, the labile factor VIII activity has  
diminished to approximately half, or less, of the original  
activity level present in freshly collected plasma. This  
makes it necessary to fractionate larger quantities of  
blood to obtain a single unit of factor VIII activity.  
25 The art has long sought a technique for enhancing the  
yield of factor VIII activity in blood plasma and partic-  
ularly in the cryoprecipitate that normally serves as the  
vehicle for administration of factor VIII to humans.

It is an object of this invention to provide a  
30 method for generating factor VIII activity from inactive  
protein.

Another object of the present invention is to  
provide a method for generating factor VIII activity in  
plasma by incubation of such material with a divalent  
35 manganese salt.

A further aspect of the present invention is to  
provide a method for generating factor VIII activity in

1 plasma cryoprecipitates by incubating the cryoprecipitate  
with a divalent manganese salt.

A still further object of the present invention  
is to provide a new product comprising a plasma cryopre-  
5 cipitate containing an effective amount for generating  
factor VIII activity of a divalent manganese salt.

The foregoing objects, together with further  
aspects of the present invention, will be apparent upon  
consideration of the following specification.

10 It has long been reported that incubation of  
normal plasma with sodium citrate, ethylene diamine  
tetra-acetic acid (EDTA) and other metal chelating anti-  
coagulants renders the plasma deficient in factor VIII  
(see Casillas et al. "Artificial Substrate for the Assay  
15 of Factors V and VIII", Coagulation, Volume 4, pages  
107-111 (1971), and J.W. Bloom et al., "A Rapid Technique  
for the Preparation of Factor V Deficient Plasma",  
Thrombosis Res., Volume 15, page 595-599 (1979)).

Because of its property as a metal chelating  
20 agent, EDTA is commonly used as an anti-coagulant in the  
collection of fresh human and animal blood. The metal  
chelating action is considered to be necessary to remove  
calcium in whose absence the blood clotting reactions do  
not occur. Whole blood is collected into anti-coagulant  
25 citrate dextrose, anti-coagulant citrate phosphate dex-  
trose, sodium citrate, and sodium oxalate. Although  
anti-coagulant heparin is not a metal chelating agent  
and acts through other mechanisms to prevent clotting,  
existing practice and regulation does not permit its use  
30 when only plasma (as contrasted with whole blood) is to be  
collected. A further drawback is that the lifespan of red  
blood cells in heparinized blood is somewhat diminished as  
contrasted with other anti-coagulant agents.

At the present time, sodium citrate is the anti-  
35 coagulant most commonly used for plasmapheresis. Exposure  
of whole blood to the presence of the above-mentioned  
metal chelating anti-coagulant agents successfully removes

1 calcium (++) and prevents clotting. However, such  
exposure also results in depleting or destroying factor  
VIII activity. For this reason, it has long been thought  
that factor VIII procoagulant activity was dependent on  
5 the presence of calcium. However, efforts to regenerate  
such factor VIII activity in whole plasma, or in plasma  
derivatives (e.g. cryoprecipitate), by the addition of  
calcium salts have been wholly unsuccessful.

It has now been unexpectedly discovered that  
10 factor VIII is a metallo-protein containing manganese and  
that manganese salts can be used to generate factor VIII  
activity from plasma proteins which do not display such  
procoagulant activity. This is surprising because  
calcium has long been considered the cation responsible  
15 for factor VIII activity. In practicing the present  
invention it is preferred to incubate a plasma cryopre-  
cipitate whose AHF activity level has been depleted by  
exposure to a metal chelating anti-coagulant, in the  
presence of a manganese salt. Suitable salts include the  
20 manganese halides, which are preferred, (i.e., chloride  
(especially preferred), bromide, iodide, fluoride),  
acetate, formate, citrate, oxalate, phosphate, nitrate,  
and sulphate. The list is not all inclusive and any  
pharmaceutically acceptable manganese salt may be employed  
25 in practicing the invention.

It has been determined that manganese salt  
incubation for factor VIII procoagulant activity synthesis  
is best conducted in the range of between about pH3 and  
pH8.5, and preferable between about pH6 and pH7.5. The  
30 temperature at which the incubation is conducted is not  
critical, and successful pro-coagulant activity synthesis  
has been obtained between about 4 and about 60 degrees  
centigrade, although optimum results have been obtained  
in the range of between about 25 and 45 degrees centigrade.  
35 As a general rule, incubation is preferably carried out at  
37 degrees centigrade. Small quantities of inorganic salt  
buffering agents such as potassium chloride and sodium

1 chloride are generally included in the incubation solution,  
but are not required. Preferably these agents are present  
in the range up to about 0.5 molar concentration. Inor-  
ganic bufferin agents including by way of non-limiting  
5 example phosphates, citrates, oxalates, barbiturates,  
imidazoles, borates, acetates, formates, succinates, and  
other pharmaceutically acceptable buffering agents of the  
type conventionally employed in plasma chemistry may also  
be present in the manganese incubated blood fraction in a  
10 similar concentration range.

Because AHF is only present in trace amounts in  
normal plasma, it would be necessary to administer large  
quantities of plasma to a patient in order to provide a  
therapeutic quantity of factor VIII. The likelihood of  
15 inducing an adverse immunologic reaction with antibodies  
carried in the patient's blood, taken together with the  
amplified risk of hepatitis attendant to such treatment,  
make it generally preferable to administer factor VIII  
in the form of a cryoprecipitate.

20 Cryoprecipitation is the principle technique em-  
ployed for the large scale production of AHF concentrates.  
In this technique, fresh frozen plasma is pooled, thawed  
at 2 degrees centigrade and the cryoprecipitate collected.  
The cryoprecipitate (a viscous liquid material) is gener-  
25 ally extracted with a buffer (of the type described above)  
sometimes followed by adsorption with aluminum hydroxide  
to remove prothrombin and other contaminants. Alterna-  
tively, an AHF fraction may be obtained from plasma  
using solid phase polyelectrolyte resins. High purity  
30 concentrates of factor VIII may be produced from cryopre-  
cipitate extracts by fractional precipitation with poly-  
ethylene glycol (PEG), PEG and glycine, and ethanol. In  
general, between about 200 and 400 units (by activity) of  
intermediate purity AHF concentrate may be recovered  
35 from a liter of blood, while between 100 and 250 units per  
liter of high purity concentrates can be obtained. The  
pro-coagulant activity of these concentrates (which is

1 substantially lost when contrasted with the higher levels  
of AHF activity initially present in the plasma from which  
they were obtained) may be substantially restored and  
enhanced by incubation with manganese salts according to  
5 the present invention. Using the present invention,  
effective AHF activity levels have been restored to  
cryoprecipitate samples in which the AHF activity level  
has been completely lost (i.e. to 0 units of activity by  
exposure to metal chelating agents). Addition of manga-  
10 nese salts to plasma, plasma fractions or cryoprecipitate,  
and incubation under the temperature and pH conditions  
described above can also be used to increase the factor  
VIII activity yield from current levels (between about  
40-60 percent activity) by a factor of approximately 50  
15 percent to levels between about 70-90 percent activity.

The manganese restoration principle of the  
present invention has been confirmed by ESR (electron spin  
resonance spectroscopy) and may be widely applied to other  
aspects of factor VIII blood chemistry. Thus, manganese  
20 + + salts can be used as an adjuvant to the anti-coagulant  
compositions presently employed in the collection of whole  
blood to minimize the loss of labile factor VIII activity.  
In this aspect of the invention Mn + + salts in the range  
of 0.001 - 0.5 molar may be added to the anti-coagulant  
25 (e.g., sodium citrate) solution present in the whole blood  
collection container.

Factor VIII has also been implicated as being  
useful in the treatment of VonWillebrands disease. While  
the exact composition of the protein component of the  
30 factor VIII molecule is not available, the EDTA treated  
(pro-coagulant inactive) cryoprecipitate has been separ-  
ated into two fractions (low molecular weight and high  
molecular weight proteins). The high molecular weight  
fraction has been identified as VonWillebrands factor. By  
35 coupling the process of the present invention with  
metal chelating treatments (such as EDTA) of factor VIII,  
it is possible to separate the relatively low molecular

1 weight coagulant inactive protein from the high molecular  
weight VonWillebrand factor by affinity chromatography,  
gel filtration, dialysis, high pressure liquid chromato-  
graphy (HPLC) or Amicon type filtration. The low molec-  
5 ular weight factor VIII protein may be incubated with  
manganese salts to synthesize a pro-coagulant active  
factor VIII metallo protein. This provides more potent  
factor VIII per unit of protein employed for therapeutic  
purposes and reduces the risk of hepatitis and immunologi-  
10 cally negative reactions.

The present invention makes it possible to  
employ EDTA or other chelating agents to destroy contami-  
nating viruses such as hepatitis often present in plasma  
fractions. The factor VIII pro-coagulant activity  
15 destroyed by exposure to EDTA may be regenerated by  
incubation with manganese salts to afford a factor VIII  
product that has a significantly lower risk of hepatitis  
attendant to its clinical use.

The technique for cryoprecipitation of whole  
20 blood for the production of AHF concentrates are discussed  
in detail in articles by: A.J. Johnson et al, Thromb.  
Diath. Haemorrh. Suppl. 35, 49 (1969); J. Newman et al.,  
British Journal of Hematology, Volume 21, page 1 (1971),  
and H.L. James and M. Wickerhouser, Vox Sang, 23, 402  
25 (1972). The technique for obtaining AHF (Factor VIII)  
fractions from plasma using solid phase polyelectrolyte  
resins are discussed in detail in an article by A.J.  
Johnson et al., J. Lab. Clin. Med., Volume 92, pp. 192-210  
(1978).

30 The principle upon which the present invention  
operates is believed to be that EDTA and other metal  
chelating anti-coagulant agents decompose factor VIII by  
chelating manganese ++ from the factor VIII molecule  
thereby destroying its pro-coagulant activity. In total  
35 plasma and its fractions, the removal of manganese ++  
renders the materials clinically deficient with respect to  
factor VIII activity.



1           The invention will be further described with  
reference to the following examples:

Example I

5           A purified human factor VIII (cryoprecipitate)  
having an activity of 1.5 units per milliliter was incu-  
bated in solutions containing respectively 45 millimolar  
EDTA, 45 millimolar EGTA and water, at room temperature  
overnight. The resulting solutions were dialyzed four  
hours in .34% citrate, pH7.4 and assayed for clotting  
10 activity.

	<u>U/ml</u>	<u>% activity remaining</u>
EDTA	nil	0
EGTA	0.42	30
H <sub>2</sub> O	1.38	91

15           The results of this test show that there is a  
substantial loss of factor VIII activity attendant upon  
incubation with EDTA and less with EGTA.

Example II

20           An attempt was undertaken to regenerate factor  
VIII activity by incubating the inactive EDTA treated  
material of Example I with manganese (++) chloride salts  
at 4 degrees centigrade for 48 hours. The result of this  
assay (summarized in the table below) revealed that  
manganese chloride is effective in regenerating lost  
25 factor VIII activity.

	<u>Salt</u>	<u>mM Conc.</u>	<u>F VIII Activity</u> <u>U/ml</u>
	MnCl <sub>2</sub>	17	1.10
30	MnCl <sub>2</sub>	8.5	0.65
	H <sub>2</sub> O	--	nil

Example III

35           The EDTA human treated factor VIII described  
above (Example I) was tested in a conventional one stage  
PTT clotting assay, and in thrombin activation clotting  
assay. The assays verified the complete loss of factor  
VIII activity. Overnight incubation of the inactive

1 material with calcium chloride (0.02 molar) at 4 degrees  
centigrade had no effect on clotting activity. A signifi-  
cant increase in clotting activity from 0 to 0.25 units  
per milliliter was obtained by overnight incubation with  
5 manganese chloride (0.02 molar) at 4 degrees centigrade.  
This regenerated factor VIII activity was increased to 17  
units/milliliters by activation with 10 ng/ml of thrombin.

Conditions: EDTA treated human factor VIII was  
incubated in 17 mM metal salts at 4 degrees centigrade  
10 overnight. Thrombin activation was carried out using  
10ng/ml purified bovine thrombin, 37 degrees centigrade  
for 1 min., at which time an aliquot was removed for  
clotting assays.

	<u>Salt</u>	<u>Clotting, U/ml</u>	<u>Thrombin Activation</u>
15	MnCl <sub>2</sub>	0.25	17
	CaCl <sub>2</sub>	nil	nil
	H <sub>2</sub> O	nil	nil

The results of this test comprise evidence that  
EDTA acts to chelate manganese from active factor VIII to  
20 render the protein inactive. Subsequent synthesis to  
active factor VIII metallo-protein follows incubation with  
a manganese (++) salt, preferably manganese chloride  
(MnCl<sub>2</sub>).

#### Example IV

25 The following test was conducted to obtain a  
partial time course of regeneration of lost factor VIII  
activity. EDTA human factor VIII (Example I) as dialyzed  
in citrate buffer and incubated with 17 millimolar (0.017  
molar) manganese chloride at different temperatures.  
30 Aliquots were removed at timed intervals and assayed for  
clotting activity and thrombin activation. Parallel  
assays were run at room temperature and 37 degrees centi-  
grade. The results of the assays are presented in  
the table VI below.

TABLE VI

Incubation Time, Min.	<u>Room Temp. Incubation</u>		<u>37 Degrees Incubation</u>	
	<u>Units/ml</u>	<u>Units/ml, Thrombin Activation</u>	<u>Units/ml</u>	<u>Units/ml, Thrombin Activation</u>
0	nil	nil	nil	nil
10	nil	nil	.06	.43
30	nil	nil	.12	.73
40	.04	.20	.14	.73
60	.06	.21	.17	.94
80	.07	.36	.17	1.04

The results of these tests indicate that synthesis of factor VIII pro-coagulant activity from pro-coagulant inactive protein occurs more rapidly at 37 degrees centigrade. Within an 80 minute incubation period, activity rose from 0 to 0.7 units per mililiter. This material was thrombin activatable to 1.04 units per mililiter. Synthesis was slower at room temperature. A control experiment using water instead of manganese chloride provided no generation of activity.

Example V

Purified bovine factor VIII (obtained according to the procedure of Schmer et al in "The Isolation and Characterization of Bovine Factor VIII", Journal of Biological Chemistry Volume 247 pp. 2512-2421 (1972)) having an activity level of 3.3 units per mililiter and being eightfold thrombin activatable was incubated with 27 milimolar EDTA at 4 degrees centigrade overnight and dialyzed against 0.34% citrate buffer (pH7.4). Clotting assays revealed that the EDTA treated factor VIII had completely lost its activity and was not thrombin activatable. Clotting activity was generated from 0 to 1.4 units per mililiter by incubating with manganese chloride (0.02 molar) at 37 degrees centigrade for 80 minutes. Aliquots of the manganese chloride incubated material were withdrawn at timed intervals and assayed for clotting activity. The generated factor VIII was thrombin acti-

1 vatable to 27 units per milliliter.

	Incubation Time, Min.	Clot Units U/ml	Thombin Activation U/ml
	0	nil	nil
5	10	.86	9.5
	20	.90	12.6
	40	.99	16.7
	50	1.10	17.2
	60	1.30	22.5
10	80	1.40	27.0

A control experiment using water instead of manganese chloride provided no generation of activity.

#### Example VI

15 A commercially obtained sample of lyophilized factor VIII concentrate (New York Blood Center) (Activity = 1.30 U/ml) was dissolved in water. Aliquots of this material were incubated for one hour at 37°C with small amounts of water, and solutions of  $MnCl_2$  and  $CaCl_2$  (both giving final concentrations of 0.02 Molar). Factor  
20 VIII procoagulant clothing activities were measured as shown below.

		mg/ml Protein Concentration	U/ml Clot Activity	U/ml Net Change In Activity
25	Sample + Adjuvant			
	comm'l factor VIII + $H_2O$	22.1	1.20	-.10
	" " " + $CaCl_2$ (20mM)	22.1	1.40	+.10
30	" " " + $MnCl_2$ (20mM)	22.1	2.20	+.90

Similar results were obtained from the cryoprecipitate derived from frozen and thawed normal human blood plasma collected in citrate dextrose (activity = 0.42  
35 U/ml) and incubated for one hour at 37°C.

-12-

1			mg/ml	U/ml	U/ml
			Protein	Clot	Net Change
	<u>Sample</u>	<u>+ Added</u>	<u>Concentration</u>	<u>Activity</u>	<u>In Activity</u>
	human				
5	cryoprecipitate				
	"	+ H <sub>2</sub> O	18.1	0.40	-.02
	"	+ CaCl <sub>2</sub> (0.02M)	18.1	0.45	+.03
	"	+ MnCl <sub>2</sub> (0.02M)	18.1	0.90	+.48

10 From the foregoing, it will be seen that the present invention provides a technique for synthesizing lost factor VIII activity from various blood fractions.

The EDTA sodium salt, EGTA and purified bovine thrombin used in these examples were purchased from Sigma Chemical Company, St. Louis, Mo. One stage prothrombin  
15 time clotting assays were conducted using bovine plasma as a standard having a 1 unit per mililiter factor VIII activity.

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## WHAT IS CLAIMED IS:

1. A method for generating factor VIII pro-coagulant activity in a mammal derived plasma fraction from which said activity has been at least partially lost which comprises incubating said fraction in the presence of an effective amount for regenerating factor VIII pro-coagulant activity of a pharmaceutically acceptable divalent manganese salt.
2. The method according to claim 1 wherein said divalent metal salt comprises a manganese halide.
3. The method of claim 2 wherein said halide salt is manganese chloride.
4. The method of claim 3 which comprises incubating said blood product in the presence of said salt for a predetermined time period.
5. The method of claim 4 which comprises conducting said incubating step at a temperature of between about 4 degrees centigrade and 60 degrees centigrade.
6. The method of claim 5 which comprises conducting said incubation at between about pH3 and pH8.5.
7. The method of claim 6 which comprises conducting said incubation in the presence of a buffer solution having a concentration of up to about 0.5 molar.
8. The method of claim 7 wherein said manganese salt has a concentration range of between about 0.001 and 0.5 molar.
9. The method of claim 6 wherein said blood product comprises a cryoprecipitate.

-14-

10. The method of claim 9 which comprises obtaining said cryoprecipitate from whole blood collected in the presence of a metal chelating anti-coagulant agent.

11. The method of claim 10 wherein said anti-coagulant agent comprises sodium citrate.

12. A blood product comprising a cryoprecipitate derived from human plasma, said cryoprecipitate containing a pharmaceutically acceptable divalent manganese chloride salt in a concentration range between 0.001 and 0.5 molar.

13. The product of claim 12 wherein said salt comprises a manganese halide.

14. The product of claim 13 wherein said halide salt is manganese chloride.

15. A method for producing potent factor VIII pro-coagulant material substantially free of hepatitis contaminants which comprises:

preparing a blood plasma derived cryoprecipitate from whole blood collected in the presence of ethylene diamine tetra acetec acid,

separating said cryoprecipitate into high and low molecular weight fractions, said low molecular weight fraction containing a pro-coagulant inactive protein, and

incubating said low molecular weight fraction in the presence of an effective amount for regenerating pro-coagulant factor VIII activity of a divalent manganese salt.

16. A method of synthesizing a metallo-protein possessing Factor VIII pro-coagulant activity which comprises the steps of:

collecting mammalian blood in the presence of a metal chelating anti-coagulant,

-15-

separating plasma from the collected blood,  
fractionating the plasma by using solid phase  
polyelectrolyte resins to obtain an AHF fraction, and  
incubating the polyelectrolyte resin AHF  
fraction in the presence of an effective amount for generating  
metallo-protein having pro-coagulant Factor VIII activity of a  
divalent manganese salt.

17. A method synthesizing a metallo-protein  
possessing Factor VIII pro-coagulant activity which comprises  
the steps of:

collecting mammalian blood in the presence of  
a metal chelating anti-coagulant,  
separating plasma from the collected blood,  
freezing the plasma,  
thawing the plasma to form a cryoprecipitate  
fraction,

incubating the cryoprecipitate fraction in the  
presence of an effective amount for generating metallo-protein  
having pro-coagulant Factor VIII activity of a divalent manganese  
salt.





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# EUROPEAN SEARCH REPORT

0052874  
Application number

EP 81 10 9808

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl. 3)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
A	DE - A - 2 906 571 (L. LINCE MORA et al.)  --		A 61 K 35/14 37/14
A	GB - A - 2 012 162 (DUNCAN LEE MCCOLLESTER)  ----		
			TECHNICAL FIELDS SEARCHED (Int. Cl. 3)
			A 61 K 35/00 33/00 37/00 A 01 N 1/00
			CATEGORY OF CITED DOCUMENTS
			X: particularly relevant if taken alone Y: particularly relevant if combined with another document of the same category A: technological background O: non-written disclosure P: intermediate document T: theory or principle underlying the invention E: earlier patent document, but published on, or after the filing date D: document cited in the application L: document cited for other reasons  &: member of the same patent family, corresponding document
The present search report has been drawn up for all claims			
Place of search	Date of completion of the search	Examiner	
The Hague	15-02-1982	MARIE	

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